The following supplement accompanies the article

Identification of a female sex pheromone in *Carcinus maenas*

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Supplement. Additional data

Nuclear magnetic resonance (NMR) measurements

For NMR, samples were prepared by dissolving a UDP sodium salt standard or 200 ml of lyophilized crab urine sample in 500 ml of D_2O. All NMR experiments were carried out on a Bruker Avance II 500 MHz spectrometer using a 5 mm liquid state probe operating at frequencies of 500.1013 MHz for ^{1}H and 202.404 MHz for ^{31}P measurements. ^{1}H experiments were conducted with a p/2 pulse length of 7 ms, a relaxation delay of 4 s, and 64k scans. ^{31}P measurements were performed with a typical p/2 pulse length of 11 ms, a relaxation delay of 2 s, and 64k scans with heteronuclear proton decoupling of typically 20 kHz applied during the acquisition time. Measurements were referenced to tetramethyl-silane or 85% phosphoric acid at 0 ppm for ^{1}H and ^{31}P measurements, respectively. All samples were measured at 278K.

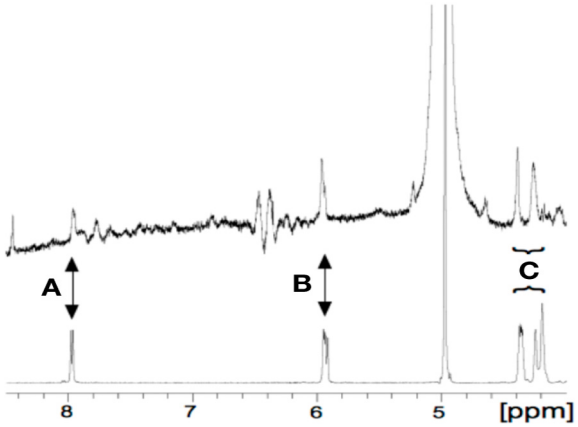


Fig. S1. *Carcinus maenas*. ^{1}H NMR spectra of the bioactive fraction obtained from crab urine (upper spectrum) and sodium salt UDP (lower spectrum) dissolved in D_2O. Peak A arises from position 6 of the uracil base, peaks labelled B are assigned to position 5 of the uracil and position 1 of the ribose sugar; the peaks labelled C arise from the remainder of the ribose protons

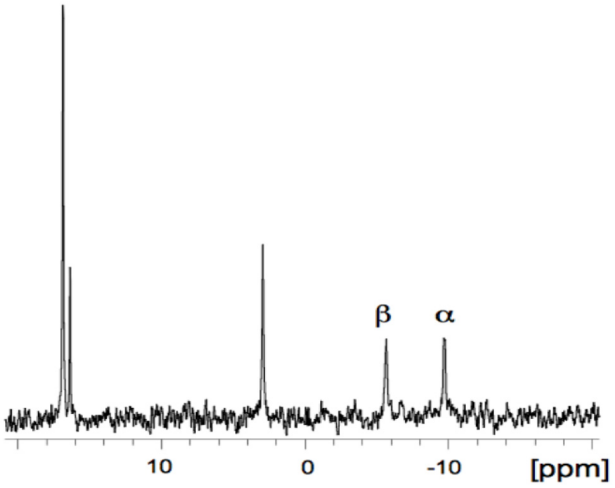
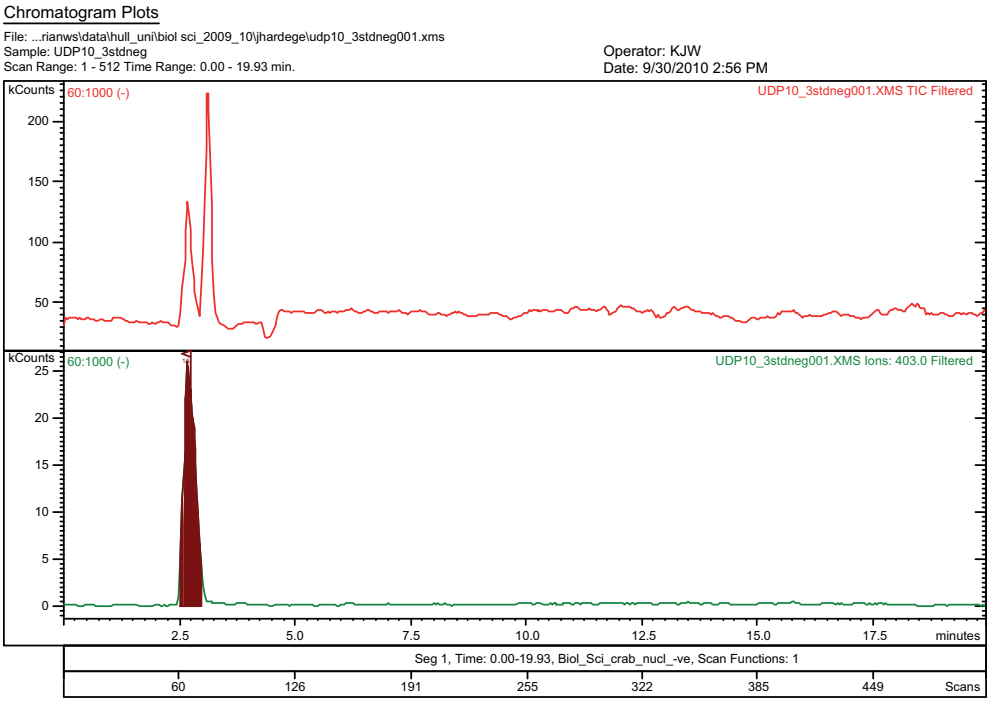


Fig. S2. *Carcinus maenas*. ^{31}P NMR spectrum of crab urine fraction dissolved in D_2O. Peaks between –4 and –10 ppm correspond to the α and β phosphates of UDP. The peak at approximately 3 ppm corresponds to free phosphates derived from the buffer. Peaks at approximately 17 ppm are unassigned. No peaks are seen at approximately –20 ppm where signals from γ phosphates would be expected to appear

Liquid chromatography-mass spectrometry (LC-MS) measurements

LC-MS analysis was undertaken using a Varian 500-MS ion trap LC-MS system with a Varian ProStar LC-212 binary solvent delivery system and a Varian ProStar 410 autosampler. The instrument was used in positive ion electrospray mode, and data were acquired and processed using the Varian MS Workstation software running on a dedicated PC-based data system. Electrospray (ESI) using nitrogen as the nebulizer gas (70.0 psi, 60°C, drying gas at 330°C, needle voltage –5 kV) producing negative ions was used (scan rate average 4.26 s scan^{-1}, data acquisition rate 0.23 Hz, multiplier offset 200 V).

a



b

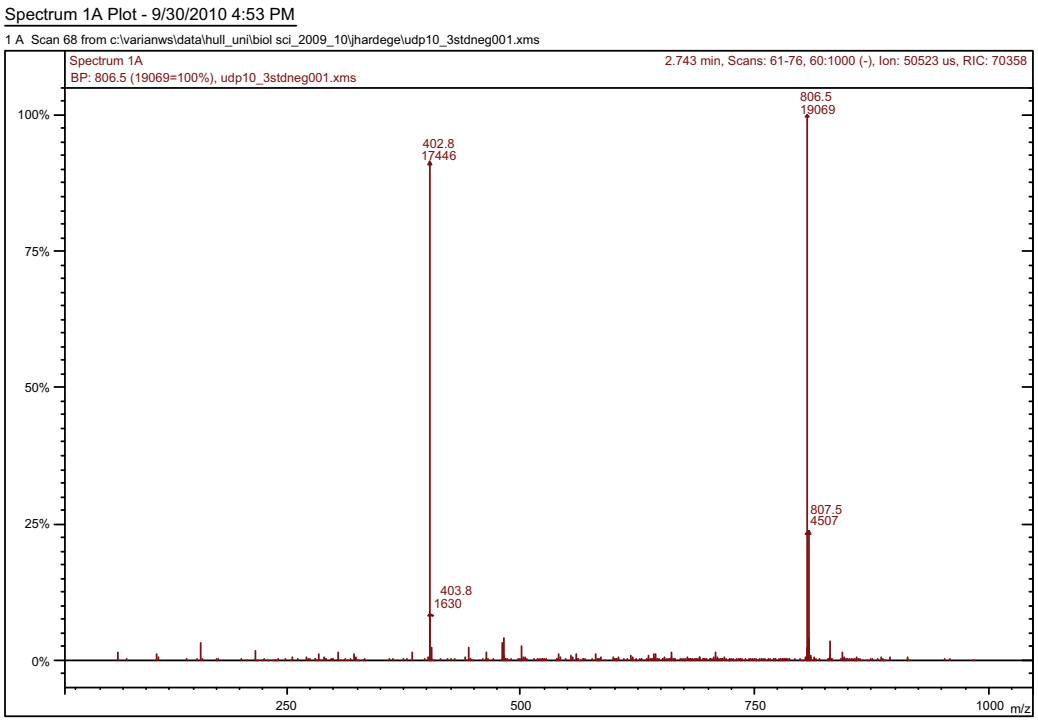
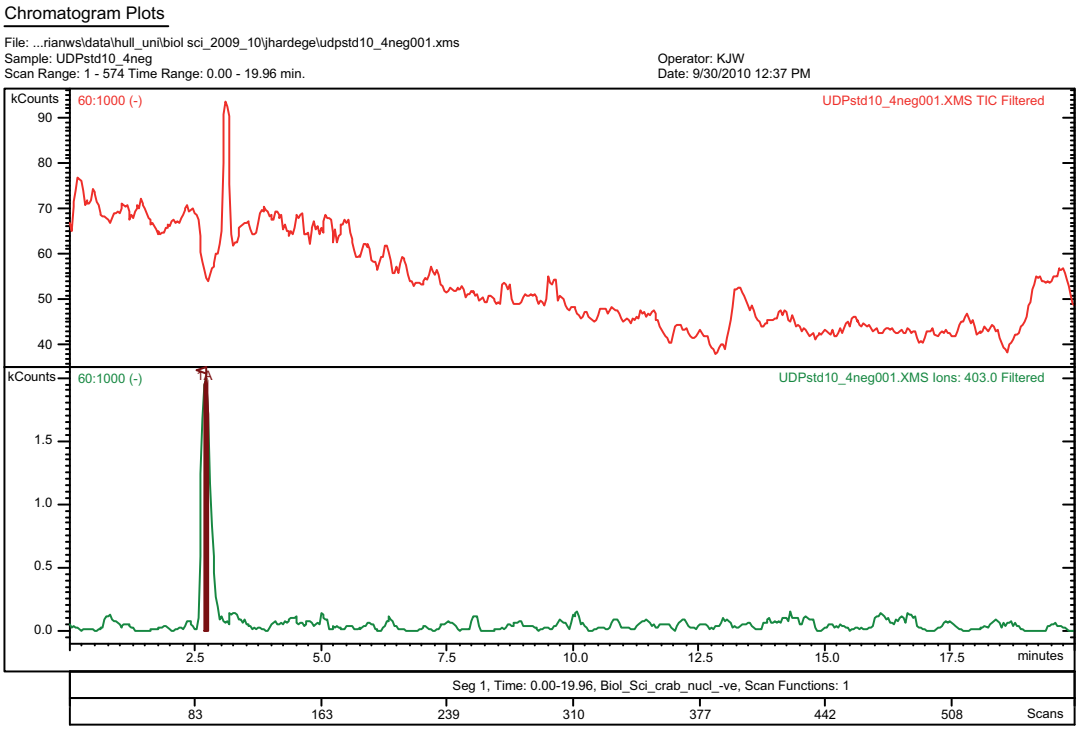


Fig. S3. Chromatogram plots of synthetic UDP at 10^{-3} M in LC-MS analysis using negative ion mode. (a) The top plot shows the total ion count and the bottom plot represents a single ion mode print of the molecular ion 402.8 that corresponds to UDP. (b) Spectrum of the peak obtained at scan no. 68 of the chromatogram plot in A. The peak at 806 m/z represents a dimer of UDP

a



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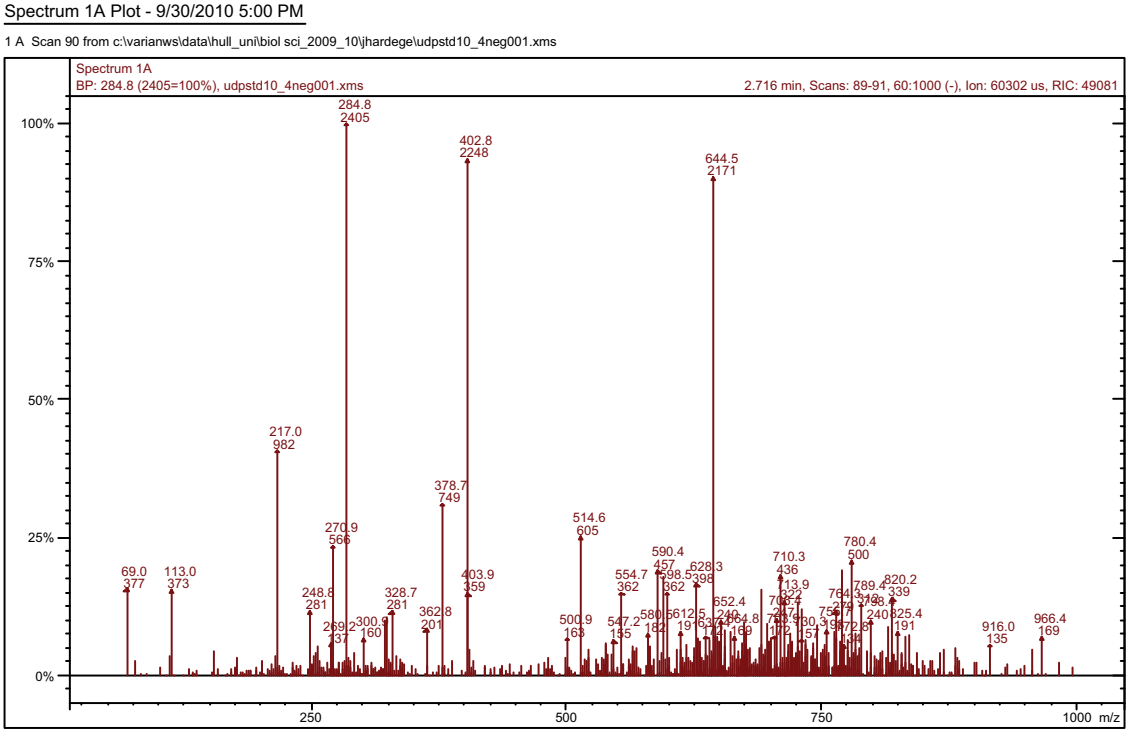
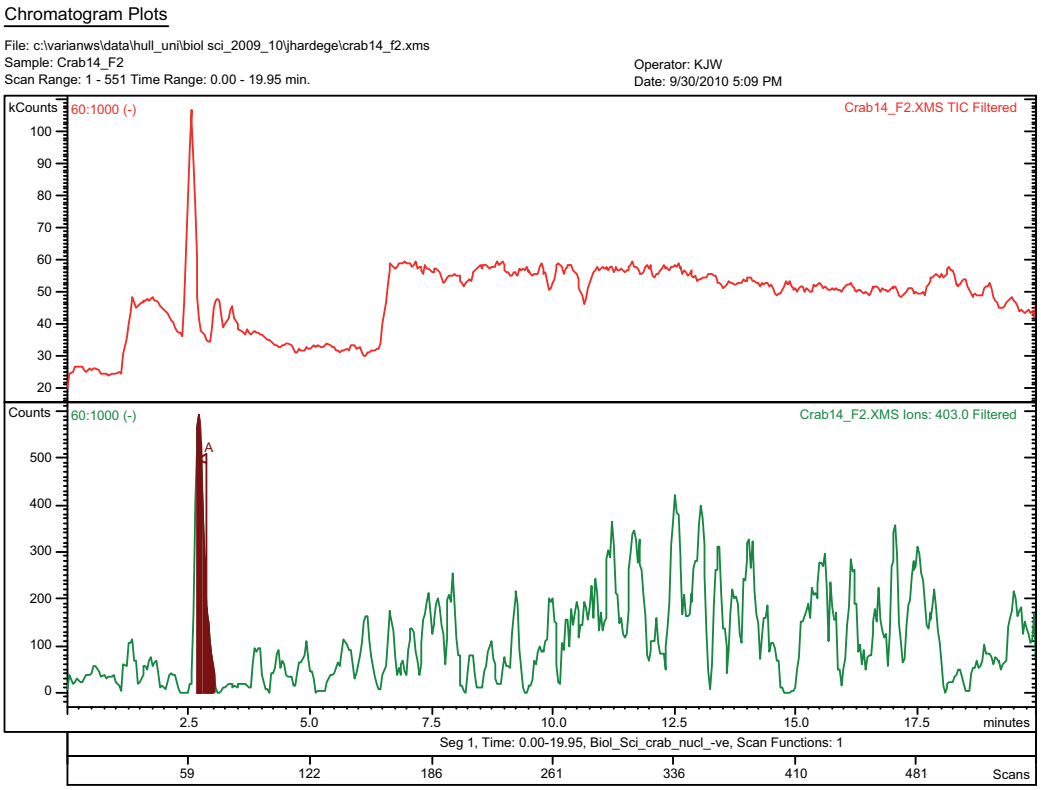


Fig. S4. Chromatogram plots of synthetic UDP at 10^{-4}M in LC-MS analysis using negative ion mode. (a) The top plot shows the TIC and the bottom plot represents a single ion mode print of the molecular ion 402.8 that corresponds to UDP. (b) Spectrum of the peak obtained at scan no. 90 of the chromatogram plot in A. The peak at 402.8 m/z is still detectable but the dimer of UDP at 806 m/z cannot be detected in the background noise, and as such, 10^{-4} M UDP represents the detection limit of UDP in samples.

a



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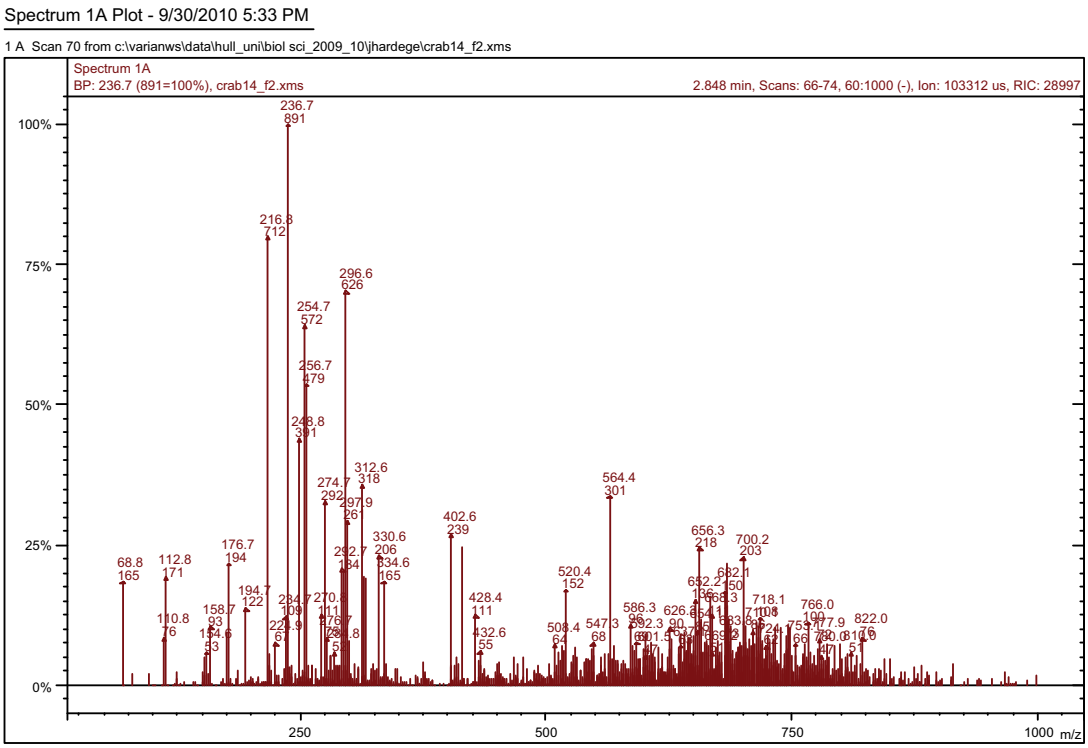


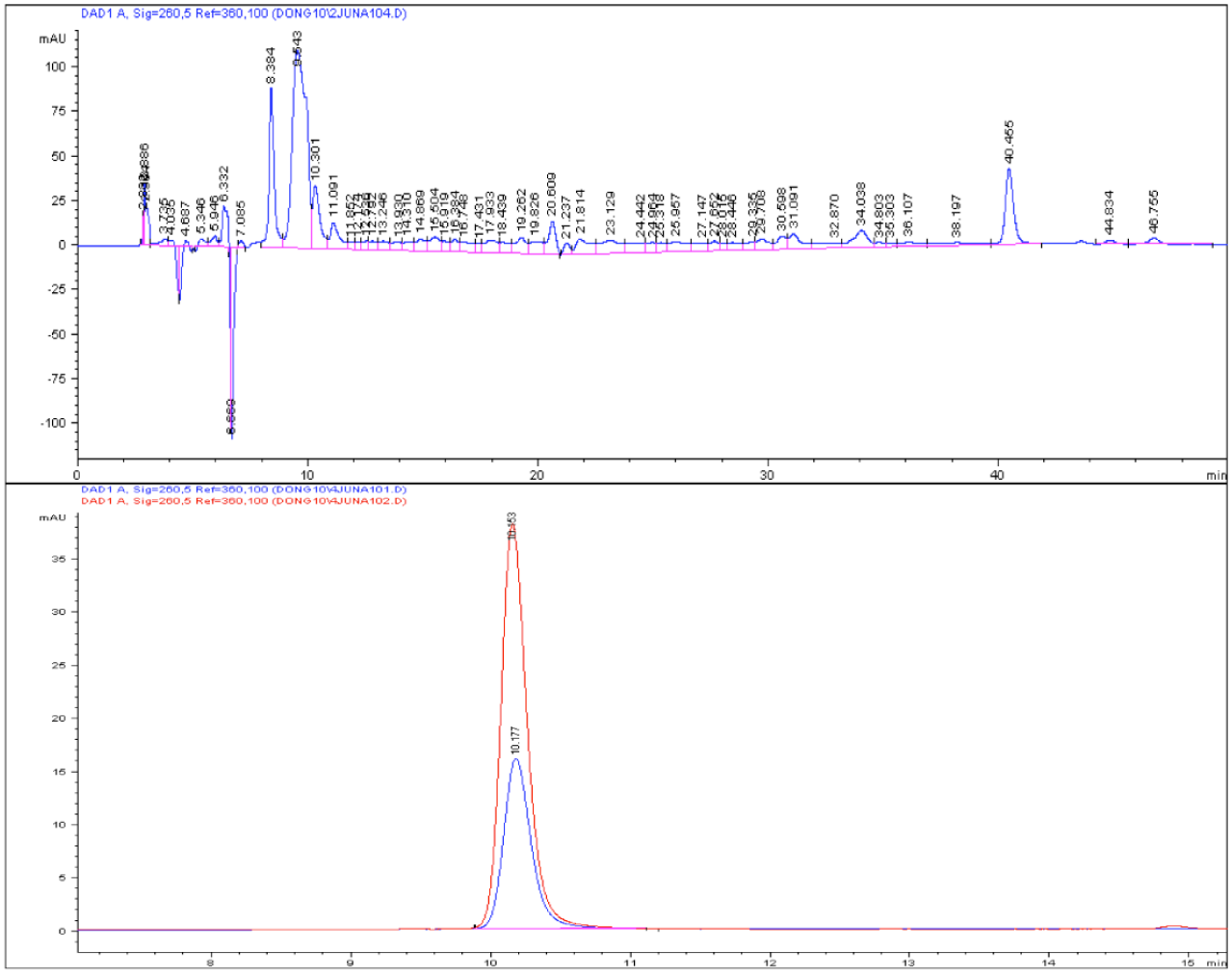
Fig. S5. *Carcinus maenas*. Chromatogram plots of the bioactive purified fraction from female urine sample in LC-MS analysis using negative ion mode. (a) The top plot shows the TIC and the bottom plot represents a single ion mode print of the molecular ion 402.8 that corresponds to UDP. (b) Spectrum of the peak obtained at scan no. 70 of the chromatogram plot in (a). The peak at 402.8 m/z is still detectable but the dimer of UDP at 806 m/z cannot be detected in the background noise. This confirms the presence of UDP in female urine samples at a concentration (peak intensity) in the region of 10^{-4} M UDP. Nevertheless, since the spectra show peak intensity at the lower detection threshold for UDP, such identification is only possible by comparing the data against a known synthetic standard when using single ion mode (SIM), as in (a). A full scan TIC of a sample using the data to identify an unknown compound is impossible, as the signal to noise ratio is insufficient

High-performance liquid chromatography (HPLC) data

Confirmation that the bioactive peak shown in Fig. 3 of the main article was indeed UDP was also achieved through co-injection of synthetic UDP to the bioactive peak and the use of HPLC analysis.

The sample F6 was obtained from ten 3 d post-molt female *Carcinus maenas*. The analysis made use of a TSK gel ODS-100V 4.6 by 250 mm, 5 µm particle size, HPLC column (TOSOH Biosciences). The mobile phase used was a 2 solvent system with A: 20 mM t-Butylamine pH 6.8 and B: A plus 10% methanol run at a gradient of: 0 to 100% B over 35 min, hold for 15 min, then post run back to A for 10 min to give a total run time of 50 min at a flow rate of 1 ml min^{-1} at 25°C. The HPLC detector was a DAD UV/Vis at 260 nm. Chromatograms in Fig. S6b,c shown below are from fractions collected from female *Carcinus maenas* urine following the purification procedure outlined in the Methods of the main article. HPLC data shown make use of a TSK gel ODS-100V 4.6 by 250 mm, 5 µm particle size, HPLC column (TOSOH Biosciences). The mobile phase used was a 2 solvent system with A: 20 mM t-Butylamine pH 6.8 and B: A plus 10% methanol run at a gradient of: 0 to 100% B over 35 min, hold for 15 min, then post run back to A for 10 min to give a total run time of 50 min at a flow rate of 1 ml min^{-1} at 25°C. The HPLC detector was a DAD UV/Vis at 260 nm. Chromatograms show the purified bioactive fraction co-injected with synthetic UDP.

a



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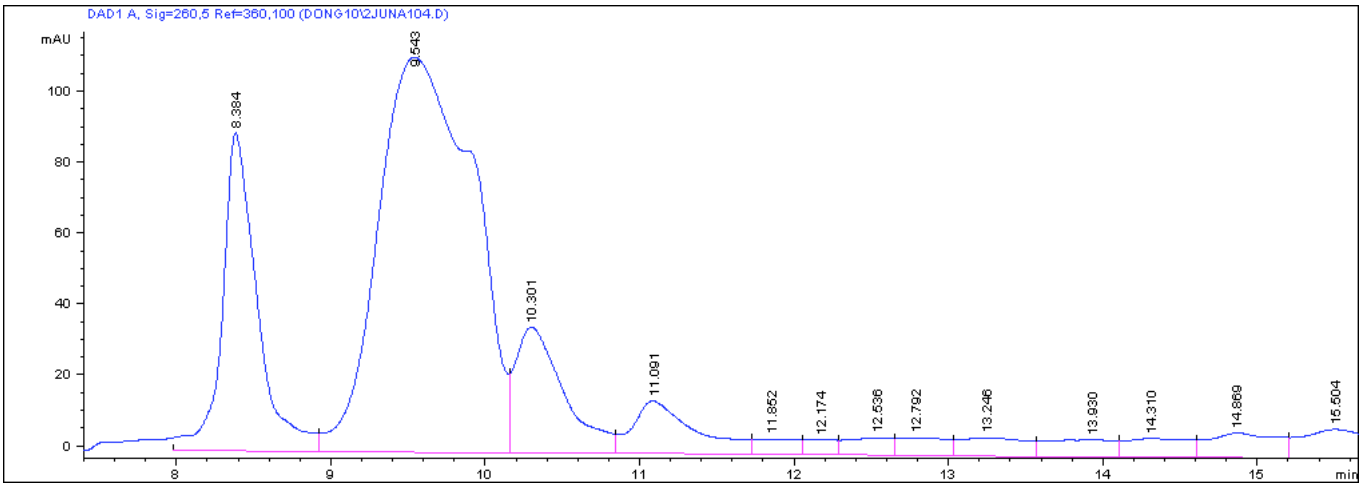

c

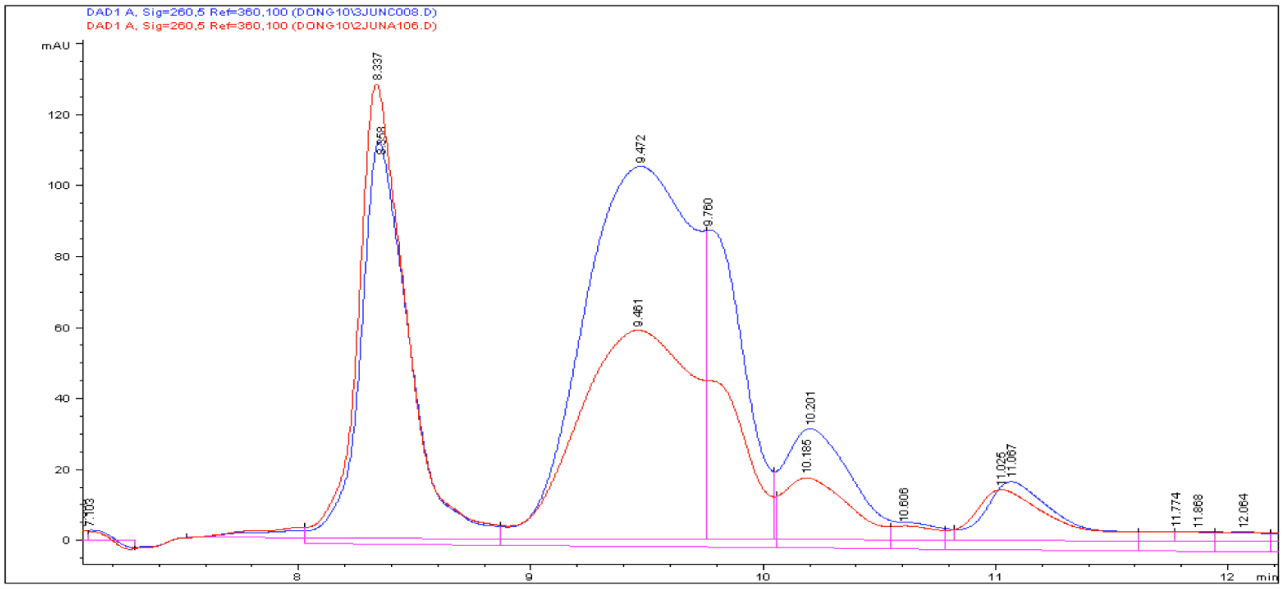

Fig. S6. *Carcinus maenas*. (a) Chromatogram showing the HPLC analysis of a bioactive female urine fraction (top) and the co-injection of synthetic UDP to the same fractionated bioactive sample (90 µl of fraction from F6 collected between 9 and 11 min + 10 µl distilled water [blue line], overlaid with same 90 µl of the same fraction spiked with 10 µl of a 2.5×10^{-4} M UDP standard [red line]). (b) HPLC analysis of the fractionated bioactive female urine sample (7–15 min) after defrosting. The single peak shown in the main manuscript (Fig. 3) has split into dimers and tautomers. (c) The same fractionated bioactive female urine sample (red) overlaid with same sample spiked with 10 µl of 10^{-4}M UDP (blue), confirming the identity of the active fraction as UDP

Additional bioassay data

Table S1. *Carcinus maenas*. Bioactivity of female and male urine and synthetic UDP. BE: bioassay equivalents

Urine samples: females	Synthetic UDP
UDP in female urine (HPLC): 7.5×10^{-5} M; (range: 3.5×10^{-5} M to 15×10^{-5} M, SD 2.1)	
Urine per female: 0.57 ml (range: 0.15–1.9, SD 0.17)	Threshold[a]: 100 µl of 10^{-5} M = 2 µg
Bioactivity threshold[a] of urine: 10 µl	Threshold grade 2: 10 µl of 10^{-5} M
BE[b] in female sample (n = 20): 1000	BE[b] in female sample attributed to UDP: 810
Urine samples: males	
UDP in male urine (HPLC): 3.1×10^{-6} M (range: 0.1–5.5×10^{-6} M)	
Bioactivity of male urine upon sexually active males at grade 5: 0/20	
Bioactivity of male urine upon sexually active males[c]: 5/20 (blank seawater control: 1/20)	

[a]Threshold for full grasping response at grade 5 (see Hardege et al. 2002 for definition)
[b]Number of potential bioassays at grade 5 possible using the sample
[c]Threshold required for a low-grade response (Grade 2: rising on tip-toe, some searching behavior; grade 2 is not considered significantly specific for sexual behavior, see Hardege et al. 2002)